

# **Intestinal epithelial cell endoplasmic reticulum stress promotes MULT1 upregulation and NKG2D-mediated inflammation**

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## Summary

Intestinal epithelial cell-specific deletion of X-box binding protein-1, an unfolded protein response-related transcription factor, results in CHOP-dependent increased epithelial cell surface expression of specific natural-killer group 2 member D (NKG2D)-ligands. This activates NKG2D-expressing intraepithelial group 1 ILCs and promotes small intestinal inflammation.

## Abstract

Endoplasmic reticulum (ER) stress is commonly observed in intestinal epithelial cells (IEC) and can, if excessive, cause spontaneous intestinal inflammation as shown by mice with IEC-specific deletion of X-box binding protein-1 (*Xbp1*), an unfolded protein response (UPR)-related transcription factor. Here, *Xbp1* deletion in the epithelium (*Xbp1<sup>ΔIEC</sup>*) is shown to cause increased expression of natural-killer group 2 member D (NKG2D) ligand (NKG2DL) murine UL16-binding protein like transcript 1 (MULT1) and its human orthologue cytomegalovirus UL16-binding protein (ULBP) via ER stress-related transcription factor C/EBP homology protein (CHOP). Increased NKG2DL expression on mouse IECs is associated with increased numbers of intraepithelial NKG2D-expressing group 1 ILCs. Blockade of NKG2D suppresses cytolysis against ER-stressed epithelial cells *in vitro* and spontaneous enteritis *in vivo*. Pharmacological depletion of NK1.1<sup>+</sup> cells also significantly improved enteritis, while enteritis was not ameliorated in recombinate activating gene 1 (*Rag1*)<sup>-/-</sup>; *Xbp1<sup>ΔIEC</sup>* mice. These studies reveal innate immune sensing of ER stress in IEC as an important mechanism of intestinal inflammation.

## **Introduction**

A defining feature of intestinal surfaces, which are subject to many types of inflammatory and neoplastic diseases (Smith et al., 2013), is the presence of a single layer of polarized epithelial cells that differentiate into distinct subtypes that function as a front-line defense of the mucosal barrier in the gut (Baker et al., 2014; Peterson and Artis, 2014). As a consequence of this anatomic location and its proximity to substantial quantities of environmental challenges, as well as the high demand for significant levels of secretory function to manage these needs, the intestinal epithelial cell (IEC) is highly dependent on the unfolded protein response (UPR) and autophagy to attenuate the significant levels of endoplasmic reticulum stress (ER stress) that occurs in order to maintain homeostasis (Bartolome et al., 2012; Grootjans et al., 2016; Jia et al., 2011; Kaser et al., 2011). Indeed, ER stress and active autophagy are demonstrable under homeostatic conditions in humans and mouse models and further increase in inflammatory bowel disease (IBD), especially in the small intestine (Bogaert et al., 2011; Deuring et al., 2013). Recent evidence shows that the UPR and/or autophagy are particularly important for mucin-secreting goblet cells and Paneth cells, which are located at the base of small intestinal crypts and secrete multiple antimicrobial peptides, as well as factors that sustain the intestinal stem cell niche (Ouellette, 2010; Salzman et al., 2010). As such, in situations of improperly folded epithelial specific proteins (Heazlewood et al., 2008) or a disabled IEC-associated UPR (Deuring et al., 2013; Kaser et al., 2008), susceptibility to colitis or spontaneous enteritis that emanates directly from the epithelium emerges (Adolph et al., 2013a; Kaser et al., 2008; Todd et al., 2008). The mechanism(s) however by which ER stress of the IEC is recognized by intestinal immune cells, and how this then is converted into intestinal inflammation is unclear.

Driven by the abundance of data on early immune recognition of diseased epithelial cells in the setting of cancer(Raulet and Guerra, 2009), we set out to investigate surface expression of major histocompatibility complex (MHC) class I and MHC class I-like proteins on ER-stressed intestinal epithelial cells. Although we did not find differences in MHC1 surface expression, we demonstrate that ER stress in IEC uregulates natural-killer group 2 member D-ligands (NKG2DL), specifically cytomegalovirus UL16-binding proteins (ULBP) in human or the orthologous murine UL-16 binding protein-like transcript 1 (MULT1; encoded by *Ulbpl1*) proteins in mouse IECs in a pathway that involves C/EBP homologous protein (CHOP; encoded by *Ddit3*), a major component of the UPR, within the IEC. We further show that upregulation of NKG2DL contributes to the development of inflammation as pharmacological blockade of the receptor for NKG2DL, NKG2D, reduces NKG2D-mediated killing *in vitro* and dampens inflammation *in vivo*. Finally, we show that NKG2D-expressing intraepithelial group 1 ILCs in particular, but not NKG2D-expressing  $\gamma\delta$  T cells, play an important role in the inflammatory response to ER stress as group 1 ILCs accumulate in response to stress in the epithelium, and their inhibition through blockade of NKG2D ameliorates ER stress-induced inflammation.

## Results

### Epithelial ER stress induces surface expression of MULT1

To address whether ER stress *per se* can induce expression of NKG2D ligands on the surface of epithelial cells, we investigated the mouse small intestinal epithelial cell line MODE-K that was transfected with a short hairpin *Xbp1* (*shXbp1*) lentiviral vector (Kaser et al., 2008). Efficiency of short hairpin knockdown was confirmed by qPCR (Fig. S1A). Interestingly, *shXbp1* MODE-K cells expressed higher levels of NKG2DL MULT1 and to a lesser extent retinoic acid early inducible-1 (*Rae1*) on their cell surface compared to control MODE-K cells (*shCtrl*), but not H60 (Fig. 1A and B). In contrast, expression of MHC-class I, which is recognized by killer inhibitory receptors, was not affected by *Xbp1* knockdown *in vitro* and knockout *in vivo* as shown with previously described *Xbp1* <sup>$\Delta^{pEC}$</sup>  mice that possess conditional deletion of X-box binding protein 1 (*Xbp1*) in the intestinal epithelium using the *Villin* (V) promoter to drive *Cre* expression (Kaser et al., 2008) (Fig. S1C and D). Further, we treated *shCtrl* and *shXbp1* MODE-K cells with the ER calcium pump inhibitor thapsigargin (Tg) to investigate the effects of acute and generalized ER stress, as opposed to specific deletion of *Xbp1*. Interestingly, treatment with Tg induced *Ulbp1* (*Mult1*), but not *Rae1*, mRNA expression in both *shCtrl* and *shXbp1* MODE-K (Fig. 1C and D). Increased *Ulbp1* mRNA expression was followed by induction of MULT1 protein surface expression (Fig. 1E). As post-transcriptional regulation of NKG2DL by microRNA binding to the 3' untranslated regions has been reported as one of the important mechanisms of NKG2D-ligand expression (Himmelreich et al., 2011; Stern-Ginossar et al., 2008), we examined *Ulbp1* mRNA stability. Importantly, *Xbp1* silencing in MODE-K cells did not affect the stability of *Ulbp1* mRNA in the presence of actinomycin-D treatment (Fig. S1B), indicating that

transcriptional induction is the mechanism of MULT1 expression on ER-stressed intestinal epithelial cells.

Next, we investigated whether NKG2DL upregulation in MODE-K cells was specific for ER stress, or could also be elicited via other stimuli such as through TLR ligands. Indeed, none of the TLR-ligands increased MULT1 or Rae1 surface expression on epithelial cells (Fig. 1F and G). Together, these *in vitro* experiments demonstrated that ER stress in intestinal epithelial cells preferentially targets the NKG2DL MULT1. We, therefore, focused our attention on MULT1 and investigated whether induction of MULT1 in response to ER stress also occurs *in vivo*. Indeed, *Ulbpl* expression was increased both in small intestinal epithelial scrapings of mice with conditional deletion of *Xbp1* in IECs (Fig. 1H) via nuclear translocation of a Cre-ER<sup>T2</sup> recombinase fusion protein after its activation with tamoxifen (T; *V-CreER<sup>T2</sup>;Xbp1<sup>fl/fl</sup>*, hereafter called '*Xbp1<sup>T-ΔIEC</sup>*'). Further proof for the upregulation of *Ulbpl* in response to epithelial ER stress *in vivo* was obtained by *in situ hybridization* (ISH) of wild type mice (*V-Cre<sup>-/-</sup>;Xbp1<sup>fl/fl</sup>*) and *Xbp1<sup>ΔIEC</sup>* mice (*V-Cre<sup>+/-</sup>;Xbp1<sup>fl/fl</sup>*), as increased mRNA transcripts were clearly visualized in intestinal epithelial cells of *Xbp1<sup>ΔIEC</sup>* mice (Figure 1I). Lastly, we confirmed that NKG2DL were also upregulated in the human intestinal epithelial cell line HT29. In line with the data obtained in mice, Tg-induced ER stress in HT29 cells selectively induced UL16-binding protein (ULBP) - 1, -5 and -6, the human counterparts of MULT1, both on mRNA (Fig. 2A) and protein levels (Fig. 2B), indicating that ER stress-induced upregulation of NKG2D ligands is a conserved across mammalian species. In support of this, *ULBPs* were also induced in other human epithelial cell lines exposed to ER stress, including a gastric (AGS), esophageal (EC-G110) and hepatic (Huh7) cancer cell line (Fig. 2C-E), as well as in cell lines from lymphoid origin,

including a B acute lymphoblastic leukemia (BALL) cell line (Fig. 2F) and T lymphocyte derived Jurkat cells (Fig. 2G).

### **CHOP binds the *Ubp1* promoter to increase *Ubp1* transcription.**

We investigated the mechanisms of *Ubp1* transcription in ER-stressed cells. Consistent with previous studies (Kaser et al., 2008), we confirmed the activation of inositol requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ; encoded by *Ern1*) and the RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) pathway as shown by increased activating transcription factor 4 (ATF4) and CHOP expression in primary intestinal epithelial cells from *Xbp1*<sup>T-*ΔIEC*</sup> mice (Fig. S1E). To determine which of these factors was associated with MULT1 induction, *shCtrl* or *shXbp1* MODE-K cells were co-silenced for IRE1 $\alpha$  (*siErn1*), ATF4 (*siAtf4*), or CHOP (*siDdit3*) and MULT1 expression was analyzed by flow cytometry. The efficiency of siRNA silencing was determined by immunoblotting and qPCR (Fig. S1F and G). *siDdit3*, but not *siErn1* or *siAtf4*, transfection of *shXbp1* MODE-K cells reversed the increased MULT1 protein and mRNA expression (Fig. 3A and 3B) observed in *shXbp1* MODE-K cells, suggesting that CHOP is involved in the induction of MULT1. Because CHOP functions as a transcription factor, we examined whether CHOP could directly regulate *Ubp1* transcription. The 5'-flanking region (–267 to –257) of the *Ubp1* gene has a similar sequence to a known CHOP-binding element, PuPuPuTGCAAT(A/C)CCC (Ubeda et al., 1996). Chromatin-immunoprecipitation (ChIP) with an anti-CHOP antibody revealed evidence of increased direct CHOP binding to the promoter region of *Ubp1* in *shXbp1* and Tg-stimulated MODE-K cells (Fig. 3C). A reporter assay using luciferase reporter vectors was created to investigate the 5'-flanking region (–1464 to 0) of the *Ubp1* gene and demonstrated that a region between –447 and 0 contained the majority of

CHOP-induced transcriptional activation of *Ubp1* (Fig. 3D). To confirm this mechanism for MULT1 induction *in vivo*, *Ddit3*<sup>-/-</sup> mice were crossed with *V-cre*<sup>+</sup>;*Xbp1*<sup>fl/fl</sup> to generate *Ddit3*<sup>-/-</sup>;*V-cre*<sup>+</sup>;*Xbp1*<sup>fl/fl</sup> mice (*Ddit3*<sup>-/-</sup>;*Xbp1*<sup>ΔIEC</sup>). Whereas *Ubp1* mRNA expression was increased in crypt epithelial cells from *Xbp1*<sup>ΔIEC</sup> mice in comparison to their littermate wild-type controls, upregulation of *Ubp1* mRNA expression was not observed in *Xbp1*<sup>ΔIEC</sup> mice when CHOP was deficient as observed in *Ddit3*<sup>-/-</sup>;*Xbp1*<sup>ΔIEC</sup> mice or their *Ddit3*<sup>-/-</sup> littermate controls (Fig. 3E).

### **NKG2D-dependent, ER stress-induced inflammation is innate in nature**

Given the strong and ER stress-specific upregulation of NKG2DL on epithelial cells, we hypothesized that this could be a mechanism by which ER stressed intestinal epithelial cells are recognized by immune cells expressing the NKG2DL receptor, NKG2D, to allow for selective killing of stressed cells. To test this, we first treated *Xbp1*<sup>T-ΔIEC</sup> mice with a neutralizing, non-depleting anti-NKG2D antibody and demonstrated that indeed, blockade of NKG2D-NKG2DL interactions diminished ER stress-induced inflammation (Figure 4A). We then focused on intestinal intraepithelial lymphocytes (iIEL) that have been proposed to provide for local and directed immune responses against IECs that are altered by environmental agents, infections, or neoplastic transformation (Rhodes et al., 2008). We investigated the subsets that have been described to express NKG2D, including various T cell subsets and natural killer (NK) cells (Raulet, 2003), using the gating strategy described in Fig. 4B and Fig. S2.

Although small iIELs from *Xbp1*<sup>T-ΔIEC</sup> mice did not contain higher frequencies of CD3<sup>+</sup> T cells and showed a trend towards lower overall frequencies of TCR-αβ<sup>+</sup> cells, we observed a significant increase in TCR-γδ<sup>+</sup> T cells (Fig. 4C). Since intraepithelial TCR-γδ<sup>+</sup> T cells can express NKG2D (Ono et al., 2012), we investigated NKG2D expression on TCR-γδ<sup>+</sup> T cells and



found significantly increased levels of NKG2D on TCR- $\gamma\delta^+$  T cells in *Xbp1<sup>T-ΔIEC</sup>* (Fig 4D). However, TCR- $\gamma\delta^+$  T cells did not significantly contribute to the spontaneous inflammation of *Xbp1<sup>ΔIEC</sup>*, as *Tcrd<sup>-/-</sup>;V-cre<sup>+</sup>;Xbp1<sup>fl/fl</sup>* (*Tcrd<sup>-/-</sup>;Xbp1<sup>ΔIEC</sup>*) mice, lacking TCR- $\gamma\delta^+$  T cells (Shiohara et al., 1996), did not exhibit lower levels of enteritis as compared to *Xbp1<sup>ΔIEC</sup>* (Fig. 4E). Consistent with this, transcriptional analysis of TCR- $\gamma\delta^+$  cells obtained from the intestinal epithelium of *Xbp1<sup>ΔIEC</sup>* mice did not reveal evidence of inflammatory and cytotoxic potential when compared to those isolated from wild type (Fig. S3).

We then investigated whether the adaptive immune system was at all required for the development of inflammation in response to ER stress in the small intestinal epithelium, by crossing *Rag1<sup>-/-</sup>* with *Xbp1<sup>ΔIEC</sup>* mice. Interestingly, *Rag1<sup>-/-</sup>;Xbp1<sup>ΔIEC</sup>* mice developed spontaneous enteritis that was similar to the enteritis observed in *Xbp1<sup>ΔIEC</sup>* mice (Fig. 4F.) and histological characteristics of ER stress, including hypomorphic Paneth cells and goblet cells (Fig. S4A), were similar to that observed in *Xbp1<sup>ΔIEC</sup>* mice, as previously described (Adolph et al., 2013a; Kaser et al., 2008). Moreover, both pathological severity and kinetics of onset were comparable between *Rag1<sup>-/-</sup>;Xbp1<sup>ΔIEC</sup>* mice and *Xbp1<sup>ΔIEC</sup>* mice (Fig. 4G). These results demonstrate that although the adaptive immune system may play a role in chronic ER stress-induced inflammation (Eri et al., 2011), it is not required for the development of inflammation in *Xbp1<sup>ΔIEC</sup>* mice.

### **Intraepithelial innate lymphoid cells (ILCs) contribute to the development of ER stress-induced inflammation in a NKG2D-dependent manner**

We, therefore, focused our attention on NK cells, gated on as CD45<sup>+</sup>CD3<sup>-</sup>CD19<sup>-</sup>NKp46<sup>+</sup>NK1.1<sup>+</sup> cells (Fig. 5A and Fig. S2B). This population expressed intranuclear eomesodermin (EOMES),

but not surface interleukin-7 receptor subunit alpha (IL7Ra) (Fig. S4B and C), identifying this population as intraepithelial group 1 innate lymphoid cells (ILCs) which consists of conventional NK cells and ILC1. Indeed, we observed a significant increase of the group 1 ILCs that expressed NKG2D and CD25, as a sign of their activation status, in response to ER stress (Fig. 5B and Fig. S2D). Similarly, and in line with our hypothesis that the group 1 ILCs mediate inflammation, increased frequencies of intraepithelial group 1 ILCs cells, expressing higher levels of NKG2D, were observed in *Rag1<sup>-/-</sup>;Xbp1<sup>ΔIEC</sup>* mice (Fig. 5C). Although we found evidence for the presence of intraepithelial innate-like NK1.1<sup>+</sup>CD3<sup>+</sup> T cells (Haas et al., 2009; Zeissig et al., 2007), a fraction of which expressed NKG2D, we observed that the expression levels of NKG2D did not differ between *Xbp1<sup>fl/fl</sup>* and *Xbp1<sup>ΔIEC</sup>* mice (Fig. S4D and E).

To study NKG2D-mediated killing of ER-stressed intestinal epithelial cells, we co-cultured *shXbp1* MODE-K cells with splenic NK cells, and demonstrated that pre-treatment of NK cells with a blocking anti-NKG2D antibody significantly decreased specific lysis *in vitro* (Fig. 5D). Similarly, silencing of *Ubp1* with *siUbp1* decreased NK cell-mediated specific lysis of *shXbp1* MODE-K cells, and this effect was further enhanced by co-treatment with anti-NKG2D antibody (Fig. 5E). Further, IELs from *Xbp1<sup>ΔIEC</sup>* mice were more cytolytic as they exhibited increased killing of MULT1-transfected RMA cells, a mouse T cell lymphoma cell line, in comparison to the activity observed with IELs from wild type (*Xbp1<sup>fl/fl</sup>*) mice (Fig. S5). To determine the importance of intraepithelial group 1 ILCs in the development of small intestinal enteritis *in vivo*, we treated *Xbp1<sup>T-ΔIEC</sup>* mice with antibodies to deplete NK1.1<sup>+</sup> cells, and demonstrated that this resulted in a significant depletion of intraepithelial group 1 ILCs defined as CD45<sup>+</sup>CD3<sup>+</sup>NKp46<sup>+</sup> cells (Sips et al., 2012) (Fig. 5F). Consistent with our observations in the co-culture system *in vitro*, NK1.1<sup>+</sup> cell-depleted *Xbp1<sup>T-ΔIEC</sup>* mice exhibited significantly reduced

enteritis scores as compared to isotype-matched control Ig treated animals (Fig. 5G), further demonstrating that intraepithelial group 1 ILCs, and potentially innate-like NK1.1<sup>+</sup> T cells when an adaptive immune system is present, play a key role in the development of ER stress-induced inflammation.

## Discussion

Here we demonstrate that ER stress in the intestinal epithelium drives spontaneous small intestinal enteritis, and can occur in the presence of only an innate immune system. Further, ER stress in intestinal epithelial cells is sensed by their increased surface expression of NKG2D ligands that are recognized by intestinal NKG2D-expressing intraepithelial group 1 ILCs and potentially innate-like T cells if an adaptive immune system is present.

It has long been known that iIELs in the small intestine represent a complex collection of lymphocytes, which includes TCR- $\alpha\beta^+$  T cells that are either CD8 $\alpha\alpha^+$  or CD8 $\alpha\beta^+$ , TCR- $\gamma\delta^+$  T cells, innate-like T cells (Wencker et al., 2014) and ILC, which includes NK cells and ILC1. Intraepithelial NK cells that are CD45 $^+$ CD3 $^-$ NKp46 $^+$  (Sips et al., 2012) and display interferon (IFN)- $\gamma$  production and cytotoxic activity have been described in mice (Keilbaugh et al., 2005; Tagliabue et al., 1982) and humans (Leon et al., 2003). It has also recently been recognized that ILC1 can be detected within the small intestinal epithelium (Robinette et al., 2015) and are increased in Crohn's disease in humans (Fuchs et al., 2013). Although the exact relationship between classical NK cells and ILC1s in the small intestinal epithelium remains controversial, particularly in the setting of inflammation, it is interesting to note that intraepithelial ILC1s are phenotypically similar to NK cells (Bernink et al., 2015; Spits et al., 2016). Our studies thus suggest that ER stress in IEC may be an important factor in the accumulation and function of intraepithelial group 1 ILCs that are observed in inflammatory bowel disease and potentially other inflammatory conditions.

NKG2D ligands are MHC class I-like molecules, which include mouse RAE-1, MULT1 and H60, whose human equivalents are MHC class I chain-related genes A and B (MICA and MICB) and ULBP 1-6 (Champsaur and Lanier, 2010; Lanier, 2015). As early as seven days after

tamoxifen-induced *Xbp1* deletion in IECs *in vivo*, we observed specific induction of *Ulbp1*, but surprisingly not *Rae-1* or *H60*. NKG2D ligands can be transcriptionally induced by various types of cell stress including DNA damage through ATM (ataxia telangiectasia, mutated) or ATR (ATM- and Rad3-related) protein kinases (Gasser et al., 2005), Toll-like receptors (TLRs) (Chen et al., 2011a; Ebihara et al., 2007; Hamerman et al., 2004), heat shock (Groh et al., 1996), and oxidative stress (Venkataraman et al., 2007), and have recently also been linked to ER stress in the setting of cancer (Gowen et al., 2015). In the latter study, ATF4 was identified as a critical protein involved in ULBP1 transcription and surface expression. Interestingly, ATF4 appeared to be particularly important for the induction of ULBP1, but not other NKG2DL, showing a similar specificity of ER stress-induced NKG2DL as reported here. In contrast however, we did not identify a role for ATF4 in the induction of MULT1 as deduced from silencing *Atf4* expression in *shXbp1* MODE-K cells. There are several possible explanations. In our studies we used an immortalized, but not transformed, intestinal epithelial cell line (Vidal et al., 1993) In addition, although knockdown of *Atf4* using *siAtf4* was efficient, CHOP levels were still observed to be increased in response to thapsigargin. This is consistent with the fact that although ATF4 is a critical regulator of CHOP, it has been described that the *Chop* promoter contains binding sites for ATF6 and XBP1 as well, and could in that way be induced independently of ATF4 by thapsigargin-induced ER stress (Kim et al., 2008).

NKG2D is a type II transmembrane-anchored C-type lectin-like activating receptor that is expressed by NK cells, activated CD8<sup>+</sup> T cells, NKT cells, subsets of TCR- $\gamma\delta$ <sup>+</sup> T cells, innate-like T cells and CD4<sup>+</sup> T cells (Champsaur and Lanier, 2010; Fuchs et al., 2013; Lanier, 2015)(Wencker et al., 2014). When NKG2D binds its ligands it promotes immune responses against infected or neoplastic cells, triggering cell-mediated cytotoxicity and secretion of

cytokines (e.g. interferon- $\gamma$ ) and chemokines (Champsaur and Lanier, 2010; Lanier, 2015). A recent proof-of-concept clinical trial of a single dose of blocking anti-NKG2D (NNC0142-0002) IgG<sub>4</sub> monoclonal antibody in patients with moderately to severely active Crohn's disease supports the importance of NKG2D in the pathogenesis of intestinal inflammation (Allez et al., 2016). Indeed, we found that in the setting of intestinal epithelial ER stress, NKG2D expression on immune cells and especially intraepithelial group 1 ILCs, was an important component of the innate immune activation as demonstrated by the decreased enteritis observed upon pharmacological blockade of NKG2D and depletion of NK1.1-expressing cells and the presence of inflammation in *Rag1*<sup>-/-</sup>*Xbp1*<sup>ΔIEC</sup> double mutant mice.

In summary, we show that intestinal epithelial cell-associated ER stress uniquely results in selective upregulation of MULT1 in mice and human ULBP-related protein in a pathway that involves CHOP. CHOP transcriptionally regulates these, but not other, NKG2DL's resulting in increased NKG2D-mediated epithelial cytolysis. As CHOP has also been shown to activate death receptor 5 (DR5) resulting in cell apoptosis (Lu et al., 2014), our observations further suggest that CHOP is a critical factor in linking ER stress to innate immune responses. Specifically, we demonstrate that upregulation of MULT1 is linked to the activity of intraepithelial group 1 ILCs, which promotes spontaneous enteritis in an NKG2D-mediated pathway, that might also involve innate-like T cells if an adaptive immune system is present. Nonetheless, our studies further reveal a dispensable role of the adaptive immune system in the enteritis associated with epithelial-associated ER stress. Altogether, we define a pathway by which ER stress is linked to the specific upregulation of particular NKG2DL's and suggest that the increased presence of group 1 ILCs in the epithelium during intestinal inflammation is reflective of a host immune response to ER stress at this site.



## Materials and Methods

### Mice

*Xbp1<sup>fl/fl</sup>* (129;B6;Balb/c) mice were backcrossed 8 times with C57BL/6 (B6) mice to obtain *Xbp1<sup>fl/fl</sup>* B6 transgenic mice, and *V-cre<sup>+</sup>;Xbp1<sup>fl/fl</sup>* (*Xbp1<sup>ΔIEC</sup>*) B6 mice were obtained as described before (Adolph et al., 2013b). For the transient Cre-mediated deletion of the floxed *Xbp1* gene, we mated *Xbp1<sup>fl/fl</sup>* B6 mice with *V-creER<sup>T2</sup>* B6 mice, which were obtained by backcrossing *V-creER<sup>T2</sup>* (129;B6) mice (kindly provided by Dr. Nicholas Davidson (Washington University, St. Louis) and Dr. Sylvie Robine (Institut Curie-CNRS, Paris)) (el Marjou et al., 2004), 8 generations onto the C57BL/6 background. Cre recombinase was activated by daily intraperitoneal administration of 1 mg tamoxifen (MP Biomedicals, CA) in 100ul sunflower oil for 7 days and mice were sacrificed the day after a final injection of tamoxifen. Sex- and age-matched littermate *V-cre<sup>-</sup>;Xbp1<sup>fl/fl</sup>* mice were used as controls. All mice were genotyped by PCR of genomic DNA isolated from proteinase K-digested tail skin by phenol extraction and isopropanol precipitation. Primer sequences were as described previously (Adolph et al., 2013b). The experiments were approved by the Harvard Standing Committee on Animals.

### H&E staining, PAS-Alcian blue staining, and histology

Small intestinal tissues from mice were isolated using standard techniques and stained with hematoxylin and eosin (H&E) as described (Adolph et al., 2013b). Goblet cells and Paneth cells were stained by Periodic acid-Schiff-Alcian blue (PAS-Alcian blue) staining using an Alcian Blue/PAS Staining Kit (Newcomer Supply, WI) according to the manufacturer's instruction. A semi-quantitative composite scoring system was used for the assessment of spontaneous intestinal inflammation, calculated as a sum of four histological subscores: mononuclear cell



infiltration (0: absent; normal sparse lymphocytic infiltration, 1: mild; diffuse increase in lamina propria, 2: moderate; lamina propria increased with basal localization aggregates displacing crypts, 3: severe; lamina propria with submucosal infiltration), crypt hyperplasia (0: absent, 1: mild, 2: moderate, 3: severe), epithelial injury/erosion (0: absent, 1: mild; crypt dropout or surface epithelial damage without frank erosion or ulceration, 2: moderate; focal ulceration, 3: severe; multifocal or extensive ulceration) and polymorphonuclear cell infiltration (0: absent, 1: mild; lamina propria only, 2: moderate; lamina propria infiltration with cryptitis or crypt abscesses, 3: severe; sheet-like or submucosal infiltration). Scores were multiplied by a factor based on the extent of the inflammation. Extent factor was derived according to the fraction of bowel length involved by inflammation: 1, 10%; 2, 10–25%; 3, 25–50%; and 4, 50%. The score was assessed by an expert gastrointestinal pathologist (J.N.G.) who was blinded to the genotype and experimental conditions of the samples.

#### Immunohistochemistry and in situ hybridization (ISH)

Formalin-fixed paraffin-embedded tissue sections were stained by standard immunohistochemistry using a VECTASTAIN ABC Kit (Vector Laboratories, CA) and DAB Peroxidase Substrate kit (Vector Laboratories) according to manufacturer's instructions. For RNA in situ hybridization, 5 µm formalin-fixed, paraffin-embedded tissue sections were processed using the RNAscope 2.5 HD Assay and Mm-Ulbp1 probe (target region 2 - 1472; Advanced Cell Diagnostics, Newark, CA) according to the manufacturer's instructions.

#### Cell culture

The SV40 large T-antigen-immortalized small intestinal epithelial cell line MODE-K (gift of D. Kaiserlian, Institute Pasteur) was maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, non-essential amino acid, sodium pyruvate 100 U/ml penicillin and 100 µg/ml streptomycin (DMEM10) (Vidal et al., 1993). The MODE-K cells were transduced with *Xbp1*-specific (*shXbp1*) or control shRNA lentiviral vectors and selected with hygromycin (Gibco, NY) to establish stable clones as described previously (Adolph et al., 2013b). The shCtrl and *shXbp1* MODE-K cells were co-silenced for *Ern1*, *Atf4*, *Ddit3*, or *Ubp1* using siRNA or scrambled control (Ambion, TX). The human gastric cancer cell line AGS; NCI-N87 (American Type Culture Collection, Manassas, MD, USA), as well as the human B cell leukemia cell line BALL-1; RCB0256 (Riken Gene Bank, Japan) and the human T cell leukemia cell line Jurkat; TIB-152 (American Type Culture Collection) were cultured in RPMI-1640 supplemented with 10% FCS. The human hepatocellular carcinoma cell line Huh7; JCRB0403 (Japanese Collection of Research Bioresources, Japan) was cultured in DMEM supplemented with 10% FCS, and the cell line EC-GI-10; RCB0774 (Riken Gene Bank) was maintained in HamF12 supplemented with 10% FCS. All *in vitro* experiments were performed in duplicate or triplicate.

#### Antibodies and reagents

Anti-β-actin (Sigma, MO), anti-CHOP, IRE1α (Cell Signaling Technology, MA), anti-ATF4, and anti-ATF6 (Santa Cruz Biotechnology, TX) were used for immunoblotting. Fluorescent-conjugated antibodies against CD3 (clone 17A2), CD4 (RM4-5), CD8 (53-6.7), CD19 (6D5), CD25 (PC61), CD45 (30-F11), NK1.1 (PK136), NKp46 (29A1.4), NKG2D (CX5), Ly49-C/I/F/H (14B11), TCRβ (H57-597), TCRγδ (GL-3), RORγt (AFKJS-9), EOMES (Dan11mag), H2K<sup>b</sup> (AF6-88.5), H2K<sup>k</sup> (36-7-5), MULT1 (237104), Rae1 (186107), H60 (205326), MICA/B

(159207), ULBP1 (170818), ULBP2/5/6 (165903), and ULBP3 (166510) were purchased from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), BioLegend (San Diego, CA) or R&D systems (Minneapolis, MN). Anti-lysozyme (DAKO) antibody was used for primary antibodies in immunohistochemistry. For blocking NKG2D receptor, rat anti-mouse NKG2D mAb was purified from hybridoma (clone CX5) supernatant. To efficiently deplete group1 ILCs in the intestinal intraepithelial compartment, anti-mouse NK1.1 mAb (clone PK136, Biolegend) and rabbit anti-asialo ganglio-N-tetraosylceramide (asialo-GM1) antisera (Wako Chemicals, VA) were used. Thapsigargin (Sigma) and tunicamycin (Sigma) were dissolved in DMSO as recommended by the manufacturer.

#### RNA isolation and quantitative PCR

RNA samples were extracted and purified using an RNeasy Mini Kit (Qiagen, MA), and complementary DNAs were synthesized using the SuperScriptIII (Life Technologies). Real-time RT-PCR was performed using a LightCycler 480 SYBR Green I Master (Roche) and a CFX96 Real-Time System (Bio-Rad, CA). Values were normalized to the expression of *Gapdh* for each sample. Primers used for qPCR were as follows for *Ulbp1* 5'-CTAACACAACCGGAAAGCCCCT-3' and 5'-CAGTGCTTGTGTCAACACGGA-3' or 5'-GCAGGCTGAGGTGTGTGGCC-3' and 5'-CCAGGTCCTGCAGTCGCCCT-3' (Chen et al., 2011b); *Rae1* 5'-TCCGCAAAGCCAGGGCCAAA-3' and 5'-GCTGGTAGGTGGAAGCGGGG-3' (Chen et al., 2011b); *H60* 5'-GTGTGATGACGATTTGTTGAG and 5'-ATTGATGGATTCTGGGCCATC-3' (Rabinovich et al., 2003); *Xbp1* 5'-AGCAGCAAGTGGTGGATTG-3' and 5'-GAGTTTTCTCCCGTAAAAGCTGA-3' (Wang and Seed, 2003); *Xbp1s* 5'-

ACACGCTTGGGAATGGACAC-3' and 5'-CCATGGGAAGATGTTCTGGG-3' (Iwakoshi et al., 2003); *Ddit3* 5'-CTGGAAGCCTGGTATGAGGAT-3' and 5'-CAGGGTCAAGAGTAGTGAAGGT-3' (Wang and Seed, 2003); *Ifng* 5'-TCAGCAACAGCAAGGCGAAAAAGG-3' and 5'-CCACCCCGAATCAGCAGCGA-3' (Olszak et al., 2014) ; *Gzmb* 5'-TCTTGACGCTGGGACCTAGGCG-3' and 5'-GGGCTTGACTTCATGTCCCCCG-3' ; *Il17a* 5'-CTCCCTTGGCGCAAAAGTGAGCT-3' and 5'-ATTGCGGTGGAGAGTCCAGGGT-3' ; *Nkg2d*; 5'-CGACCTCAAGCCAGCAAAGTG-3' and 5'-TGTTGCTGAGATGGGTAATG-3' . Primers for human cell line were as follows for *MICA* 5'-CCTTGGCCATGAACGTCAGG-3' and 5'-CCTCTGAGGCCTCGCTGCG-3'; *MICB* 5'-ACCTTGGCTATGAACGTCACA-3' and 5'-CCCTCTGAGACCTCGCTGCA-3'; *ULBP1* 5'-CAAGTGGAGAATTTAATACCCATTGAG-3' and 5'-TGTTGTTTGAGTCAAAGAGGA-3'; *ULBP2* 5'-TTACTTCTCAATGGGAGACTGT-3' and 5'-TGTGCCTGAGGACATGGCGA-3'; *ULBP3* 5'-CCTGATGCACAGGAAGAAGAG-3' and 5'-TATGGCTTTGGGTTGAGCTAAG-3'; *ULBP4* 5'-CCTCAGGATGCTCCTTTGTGA-3' and 5'-CGACTTGCAGAGTGGAAGGATC-3'; *ULBP5* 5'-TGGCCGACCCTCACTCTCT-3' and 5'-CCGTGGTCCAGGTCTGAACT-3'; *ULBP6* 5'-AATCTCTTGTCCCCAGCCCT-3' and 5'-GTGAGGGTCGTCTCGCCTA-3'.

#### Flow cytometry

Intestinal epithelial cells (IEC) and intestinal intraepithelial lymphocytes (iIEL) were isolated as described previously (Olszak et al., 2014). Briefly, fat and Peyer's Patches were removed from collected small intestines, and then the intestines were cut longitudinally and washed in PBS. After removing mucus by treatment with 1 mM dithiothreitol (DTT) in PBS for 15 min at room

temperature, intestines were disrupted by shaking in RPMI-1640 containing 20 mM HEPES and 5 mM EDTA for 30 min at 37 °C. After filtering through a 100 µm strainer, cells were layered on a 40–75% Percoll gradient (GE Healthcare, NJ), and IECs were isolated from the top layer and iIELs were isolated from the cells at the 40–75% interface. After filtering through a 40 µm strainer, cells were stained with fluorescent-conjugated antibodies after incubation with anti-CD16+CD32 antibody for 15 min on ice. Flow cytometry was carried out using a MACSQuant (Miltenyi Biotec, CA) and analyzed using FlowJo software (TreeStar, OR).

#### Intestinal epithelial scrapings and crypt isolation

Mice were sacrificed and the intestines were collected, opened longitudinally, and washed with ice-cold PBS. Intestinal epithelium was collected by scraping with glass slides and snap-frozen into liquid nitrogen for further analysis. For protein analysis, the intestinal epithelial scrapings were homogenized in RIPA buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor (Complete, Roche Applied Science) using a 25 G needle with a syringe. Lysates were cleared by centrifugation 10,000 x g for 15 min at 4 °C and then assayed by standard immunoblotting by using specific antibodies as indicated in the Results and Legends. For RNA analysis, RNA was extracted from the scraping by using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. To isolate crypts from mouse small intestine, collected intestine was flushed by ice-cold PBS, cut longitudinally, and then incubated on ice for 30 min in 2 mM EDTA in PBS. Two sedimentation steps and application of a cell strainer separated crypts from villi, which were then used for RNA isolation (Qiagen).

### Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) with rabbit anti-CHOP (Cell Signaling Technologies) and control IgG rabbit antibody (Cell signaling Technologies) was performed in Xbp1 and control silenced MODE-K cells by using a SimpleChIP Plus Enzymatic Chromatin IP Kit (Cell Signaling Technologies). Immunoprecipitated DNA was subject to quantitative PCR (qPCR) to determine enrichment of CHOP binding to respective promoters (–431 to –272 bp relative to the start codon of *Ulbp1*) and results were normalized to input chromatin DNA. Primers used for qPCR were as follows; forward 5'-TGTAGATCACCTACCCAGCCT-3' and reverse 5'-TAAGAAGGACTCGAAGTGCAGGA-3'.

### Reporter assay

To generate Ulbp1-pGL3 firefly luciferase reporter plasmids, DNA fragments including the *Ulbp1* promoter region –1464 to 0, –1464 to –427, and –447 to 0 bp relative to the start codon were amplified from mouse genomic DNA using primers; 5'-CCGGGGTACCTGAGAACATAGCAGAACAGACCAG-3' and 5'-CCGGGCTAGCAGCTGCTTCTCTAGACTCTGGC-3', 5'-CCGGGGTACCTGAGAACATAGCAGAACAGACCAG-3' and 5'-CCGGGCTAGCCTACAGAGCAAGCAGGAGCTC-3', and 5'-CCGGGGTACCGAGCTCCTGCTTGCTCTGTAG-3' and 5'-CCGGGCTAGCAGCTGCTTCTCTAGACTCTGGC-3', respectively. The PCR products were inserted into pGL3-Basic (Promega, WI) plasmid between the *Kpn* I and *Nhe* I site. To generate the mouse CHOP expression plasmid, cDNA encoding mouse CHOP corresponding to nucleotides 30 to 734 was amplified by PCR using primers; 5'-

ccggGAATTCcatacaccaccacacctgaa-3' and 5'-ccggAAGCTTgtaccgtctatgtgcaagc-3', and was inserted into pcDNA3.1(-) plasmid between *Eco* RI and *Hind* III. MODE-K cells were transfected with 50 ng of Ulbp1-pGL3 luciferase reporter plasmids, 10 ng of pRL-CMV plasmid (Promega) encoding Renilla luciferase, and 140 ng of mouse CHOP expression plasmid (pcDNA3.1(-)-mDdit3 or pcDNA3.1(-) using Lipofectamine 2000 reagent (Invitrogen), as recommended by the manufacturer's instruction. The reporter gene activities were measured by a Dual-luciferase reporter assay System (Promega) according to the manufacturer's instructions (Promega), 24 h after transfection. Data were normalized against Renilla luciferase activity.

#### Cytotoxic assay

Primary mouse NK cells were isolated from mouse spleens using NK cell isolation kits (Miltenyi Biotec) according to the manufacturer's instruction. The NK cells were cultured in RPMI-1640 supplemented with 10% FBS, and 200 units/mL recombinant human IL-2 (NIH) for a week. IELs were isolated as described above. In some experiments, the effector cells were pre-treated with anti-NKG2D (clone CX5) blocking antibodies or an isotype-matched control Ig for 30 minutes at 4°C. Target cells were labeled with 2 mM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) for 10 min at 37 °C. After incubation with effector cells for 4 hr at 37 °C at indicated effector:target (E:T) ratio, cells were stained with 7-aminoactinomycin D (7-AAD) (BioLegend) for 15 min and analyzed by flow cytometry. The specific cytotoxicity was calculated as (number of double positive cells for CFSE and 7-AAD)/(CFSE positive cells) X 100%.

#### Anti-NKG2D in vivo treatment and group 1 ILCs depletion experiments

*Xbp1*<sup>T-ΔIEC</sup> mice were treated with anti-NKG2D blockade antibody (CX5), or an isotype-matched control Ig (200 µg/injection) intraperitoneally on day -1 and 2 after tamoxifen injection, and small intestines were collected on day 7 after tamoxifen injection. Similarly, *Xbp1*<sup>T-ΔIEC</sup> mice were treated with 200 µg anti NK1.1 antibody (PK136) or isotype-matched Ig control, with anti-asialo-GM1 (10 µl per mouse) on day -1 of tamoxifen injection, to ensure efficient depletion of NK1.1<sup>+</sup> cells in the intraepithelial compartment of the intestine. NK1.1<sup>+</sup> cell depletion was confirmed on day 7 after administration of tamoxifen using flow cytometry.

### Statistical analysis

Statistical significance was calculated using an unpaired two-tailed Student's t-test or a Mann-Whitney *U*-test, when appropriate, and considered significant at *P*<0.05. In experiments comparing more than two groups, one-way analysis of variance (ANOVA) with Bonferroni's post-hoc testing was performed. Data were analyzed using GraphPad Prism software (GraphPad Software, Inc., CA).

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## Figure Legends

### Figure 1. ER stress results in upregulation of MULT1 in vitro and in vivo.

(A) Representative histograms of NKG2D ligands on shCtrl and *shXbp1* MODE-K cells by flow cytometry (one of two independent experiments). (B) Knockdown of *Xbp1* in MODE-K cells results in significantly increased surface expression of MULT1 and Rael as measured by increased mean fluorescent intensity (MFI) on MODE-K cells (one of two independent experiments). (C, D) Generalized ER stress, by administration of thapsigargin (Tg), similarly increases mRNA expression of *Ulbp1* (C), but not *Rael* (D) in shCtrl and *shXbp1* MODE-K (one of two independent experiments). (E) In line with this, MULT1 cell surface expression increased



significantly after Tg stimulation of shCtrl and *shXbp1* MODE-K cells. (F, G) Increase in MULT1 surface expression (F), but not Rael surface expression (G), occurs specifically in response to Tg-induced ER stress in MODE-K, but not in response to treatment with a variety of TLR ligands (one of two independent experiments). (H) *Ulbp1* mRNA expression is increased in small intestinal crypt isolations of mice with deletion of *Xbp1* (*Xbp1<sup>ΔIEC</sup>*) in IECs, as compared to wild type mice (*Xbp1<sup>fl/fl</sup>*) (n=6 and n=4, respectively). (I) Increased *Ulbp1* mRNA in small intestinal epithelial cells of *Xbp1<sup>ΔIEC</sup>* mouse compare to *Xbp1<sup>fl/fl</sup>* mouse (n=4 per group, scale bar: 100 μm on low magnification and 20 μm on high magnification). PGN; peptidoglycan, poly IC; polyinosinic-polycytidylic acid, LPS; lipopolysaccharide, Flagel; Flagellin, CpG; CpG oligodeoxynucleotides. All data represent mean ± s.e.m.

## Figure 2. ULBP upregulation by ER stress on human cell lines

(A, B) NKG2D ligands mRNA (A) and protein (B) expression in human colorectal cancer cell line (HT29) by qPCR (A) or flow cytometry (B) after MOCK or Tg stimulation for 4 hr (A) or 24 hr (B). (C-G) NKG2D ligands mRNAs expression of AGS (C), EC-GI-10 (D), Huh7 (E), BALL (F) and Jurkat (G) cell lines after MOCK or Tg stimulation for 4 hr. All data represent mean ± s.e.m. A-G: Representative plots of at least two independent experiments.

## Figure 3. CHOP binds the *Ulbp1* promotor and is important for ER stress-induced upregulation of *Ulbp1*

(A) Whereas co-silencing of IRE1α or ATF4 did not have an effect on MULT1 surface expression on *shXbp1* MODE-K cells, as compared to shCtrl, co-silencing with *siDdit3* significantly decreased MULT1 MFI (one of two independent experiments). (B) In line, *siDdit3*

significantly decreased *Ubp1* mRNA expression in MODE-K (one of two independent experiments). (C) qPCR for predicted MULT1 promoter sequence after anti-CHOP chromatin immunoprecipitation. shCtrl with/without Tg stimulation or *shXbp1* MODE-K cells were analyzed. (one of two independent experiments) (D) Firefly luciferase reporter plasmids including the *Ubp1* promoter regions with CHOP expression plasmid (pcDNA-mDdit3) or empty plasmid (pcDNA) were co-transfected into shCtrl MODE-K cells. Luciferase activity was measured and normalized against Renilla luciferase activity (one of two independent experiments). (E) *Ubp1* mRNA expression in mucosal scraping is not increased in *Ddit3*<sup>-/-</sup>; *Xbp1*<sup>AIEC</sup> double mutant mice as opposed to *Xbp1*<sup>AIEC</sup> single knockout mice (*n*=7-12 per group). All data represent mean ± s.e.m.

**Figure 4. NKG2D-dependent ER stress-induced intestinal inflammation does not require an adaptive immune system.**

(A) Development of enteritis in *Xbp1*<sup>T-AIEC</sup> mice is significantly inhibited by treatment with anti-NKG2D antibody (CX5), as compared to isotype-matched control Ig (*n*=13-14 per group). Representative H&E of experimental group. (B, C) Flow cytometric analysis of intestinal intraepithelial lymphocytes (IEL) from *Xbp1*<sup>fl/fl</sup> and *Xbp1*<sup>T-AIEC</sup> mice for CD3<sup>+</sup> cells, αβT cells and γδT cells (*n*=4 per group) (D) NKG2D expression is significantly increased on IEL-γδT cells from *Xbp1*<sup>T-AIEC</sup> mice (*n*=4 per group). (E) Enteritis scores of *Xbp1*<sup>fl/fl</sup>, *Xbp1*<sup>AIEC</sup>, *Xbp1*<sup>fl/fl</sup>; *Tcrd*<sup>-/-</sup>, *Xbp1*<sup>AIEC</sup>; *Tcrd*<sup>-/-</sup> demonstrates that TCRγδ are not required for ER stress-induced inflammation (*n*=4-9 mice per group). (F) H&E staining of 12 weeks old *Xbp1*<sup>fl/fl</sup>, *Xbp1*<sup>AIEC</sup>, *Rag1*<sup>-/-</sup>*Xbp1*<sup>fl/fl</sup>, and *Rag1*<sup>-/-</sup>*Xbp1*<sup>AIEC</sup> mice shows normal histology in *Xbp1*<sup>fl/fl</sup> and *Rag1*<sup>-/-</sup>*Xbp1*<sup>fl/fl</sup>. Enteritis is observed in both *Xbp1*<sup>AIEC</sup> and *Rag1*<sup>-/-</sup>*Xbp1*<sup>AIEC</sup> mice (representative H&E of experimental

groups). (G) Enteritis scores of *Rag1*<sup>-/-</sup>, *Xbp1*<sup>ΔIEC</sup> double mutant mice, compared to *Xbp1*<sup>ΔIEC</sup> shows similar onset and kinetics of inflammation (*n*=5-16 per group).

**Figure 5. Intraepithelial group 1 ILCs play a key role in the development of intestinal inflammation in response to ER stress.**

(A) Flow cytometric analysis of IEL group 1 ILCs from *Xbp1*<sup>fl/fl</sup> and *Xbp1*<sup>T-ΔIEC</sup> mice. (B) NKG2D and CD25 expression on IEL group 1 ILCs in *Xbp1*<sup>fl/fl</sup> and *Xbp1*<sup>T-ΔIEC</sup> mice (*n*=4 mice per group). (C) Frequency of IEL group 1 ILCs in *Xbp1*<sup>fl/fl</sup>; *Rag1*<sup>-/-</sup>, *Xbp1*<sup>ΔIEC</sup>; *Rag1*<sup>-/-</sup> (*n*=4 mice per group). (D) CFSE-labeled MODE-K cells were incubated for 4 hr with primary splenic NK cells pre-activated by recombinant IL-2. Cytotoxicity was assessed by flow cytometry after 7-AAD staining. NK cells were pretreated with anti-NKG2D blocking antibody (CX5) or isotype-matched control Ig for 30 min before incubation at indicated effector:target (E:T) ratios. (Depicted is one of two independent experiments) (E) NK cells, pretreated with anti-NKG2D (CX5) or isotype-matched control Ig, were used as effector cells. shCtrl and *shXbp1* MODE-K cells co-silenced with *Ubp1* or control siRNA were used as target cells. E:T ratio was 16:1 (Depicted is one of two independent experiments) (F) Flow cytometry of CD3-NKp46+ IEL shows efficient depletion of IEL group 1 ILCs (*n*=4 per group) (G) Depletion of IEL group 1 ILCs inhibits the development of inflammation in of *Xbp1*<sup>T-ΔIEC</sup> mice (*n*=8-9 mice per group). Representative H&E staining of indicated treatment. All data represent mean ± s.e.m.

**Figure. 1**

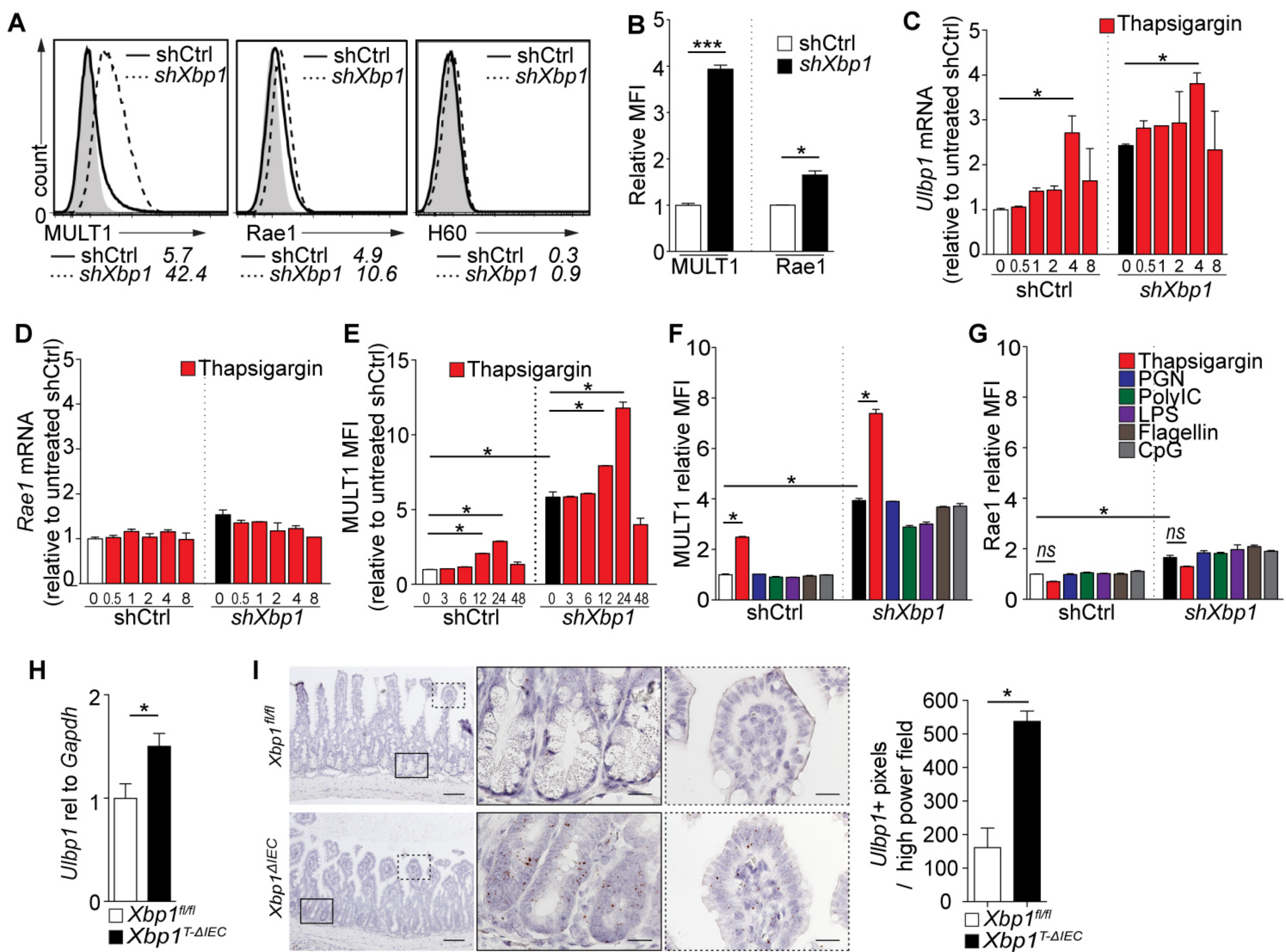




Figure. 2

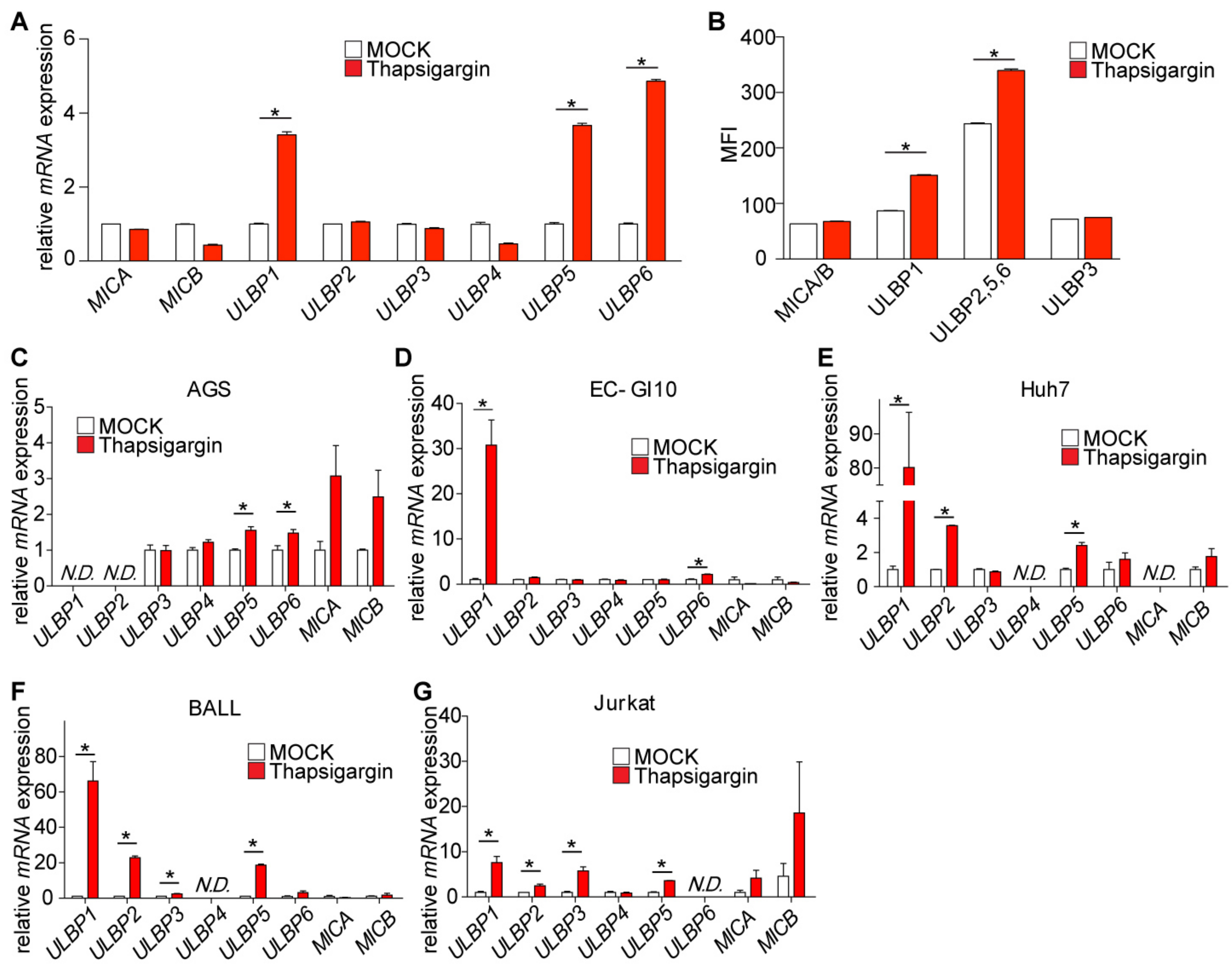


Figure. 3

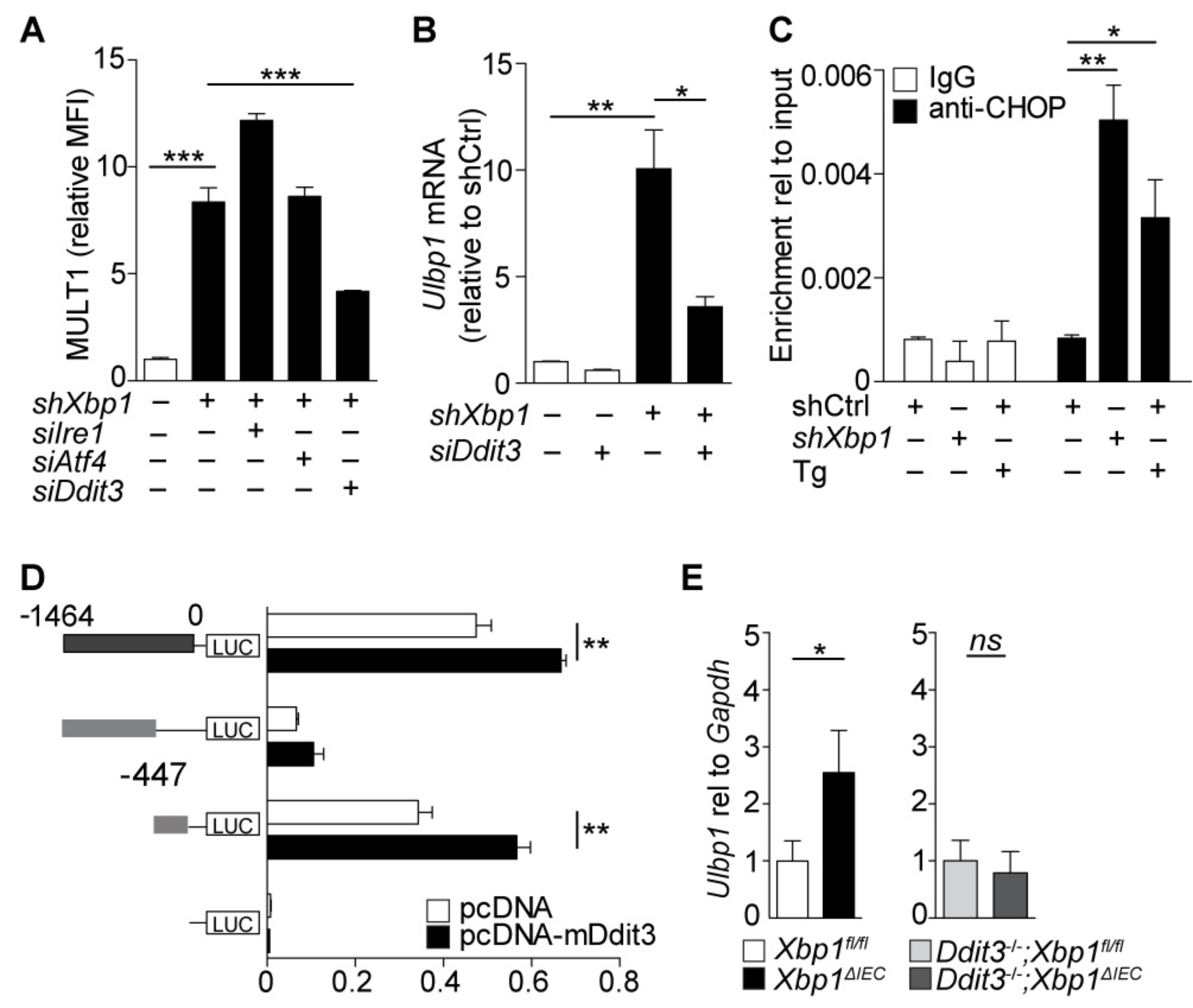




Figure. 4

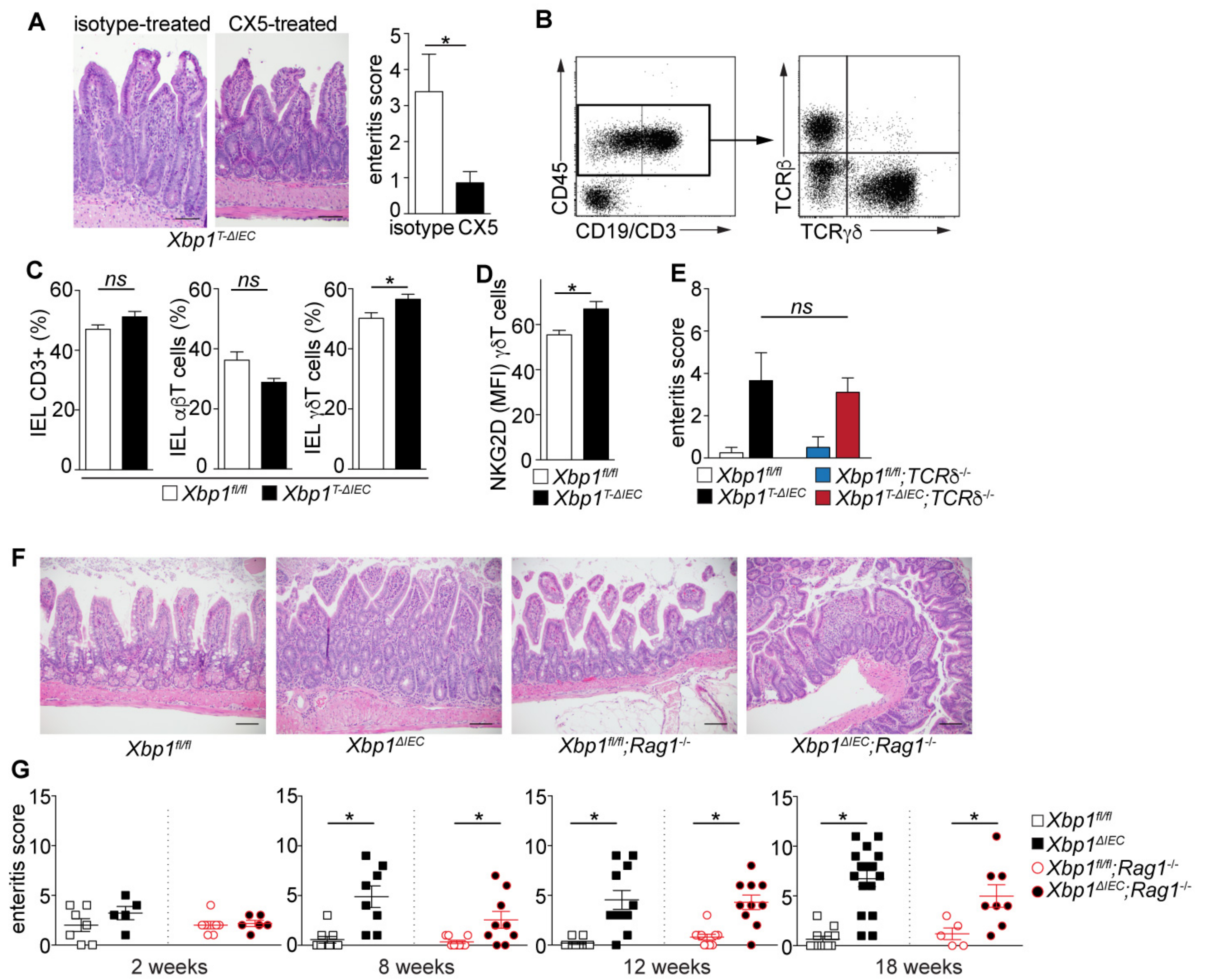
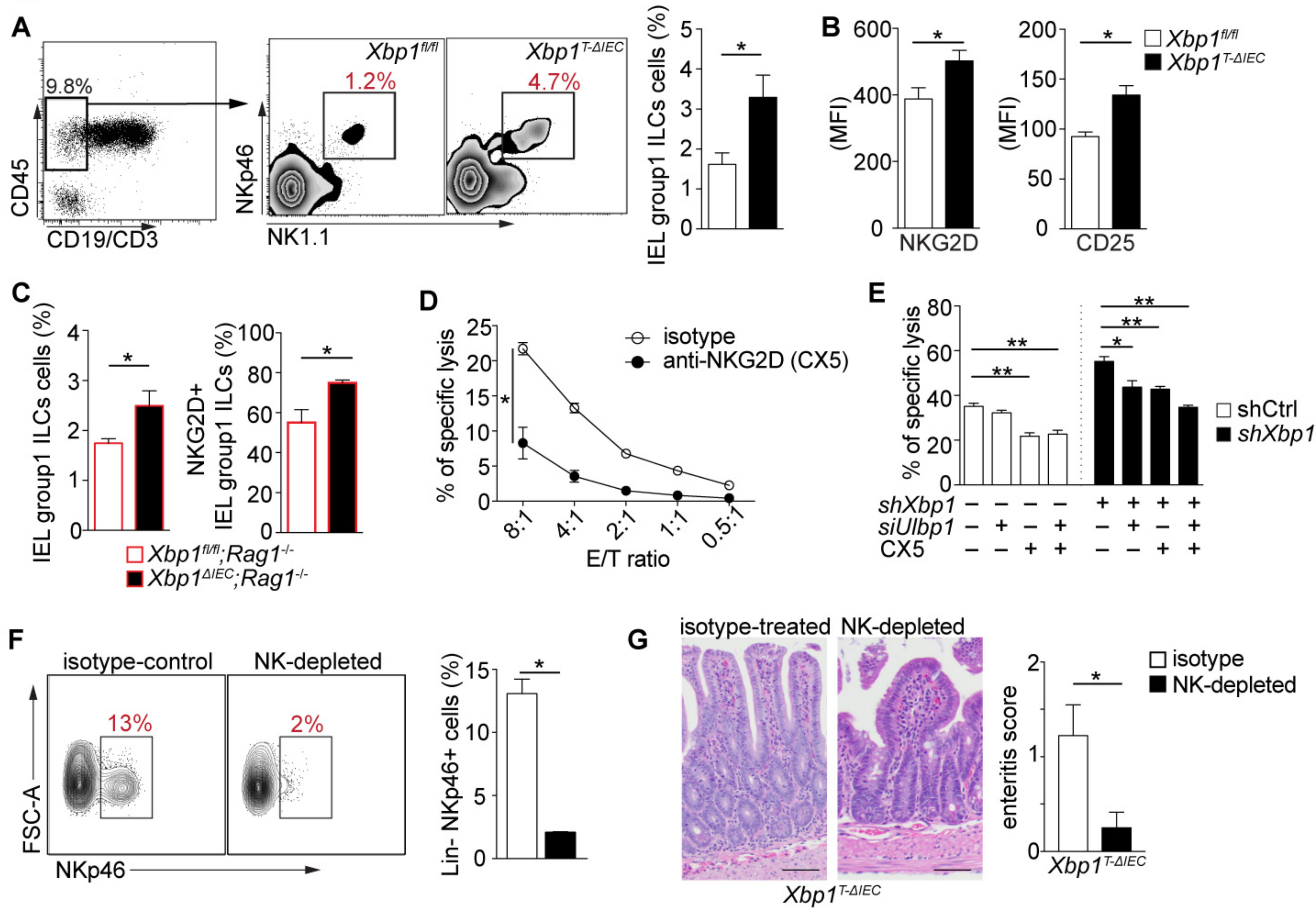


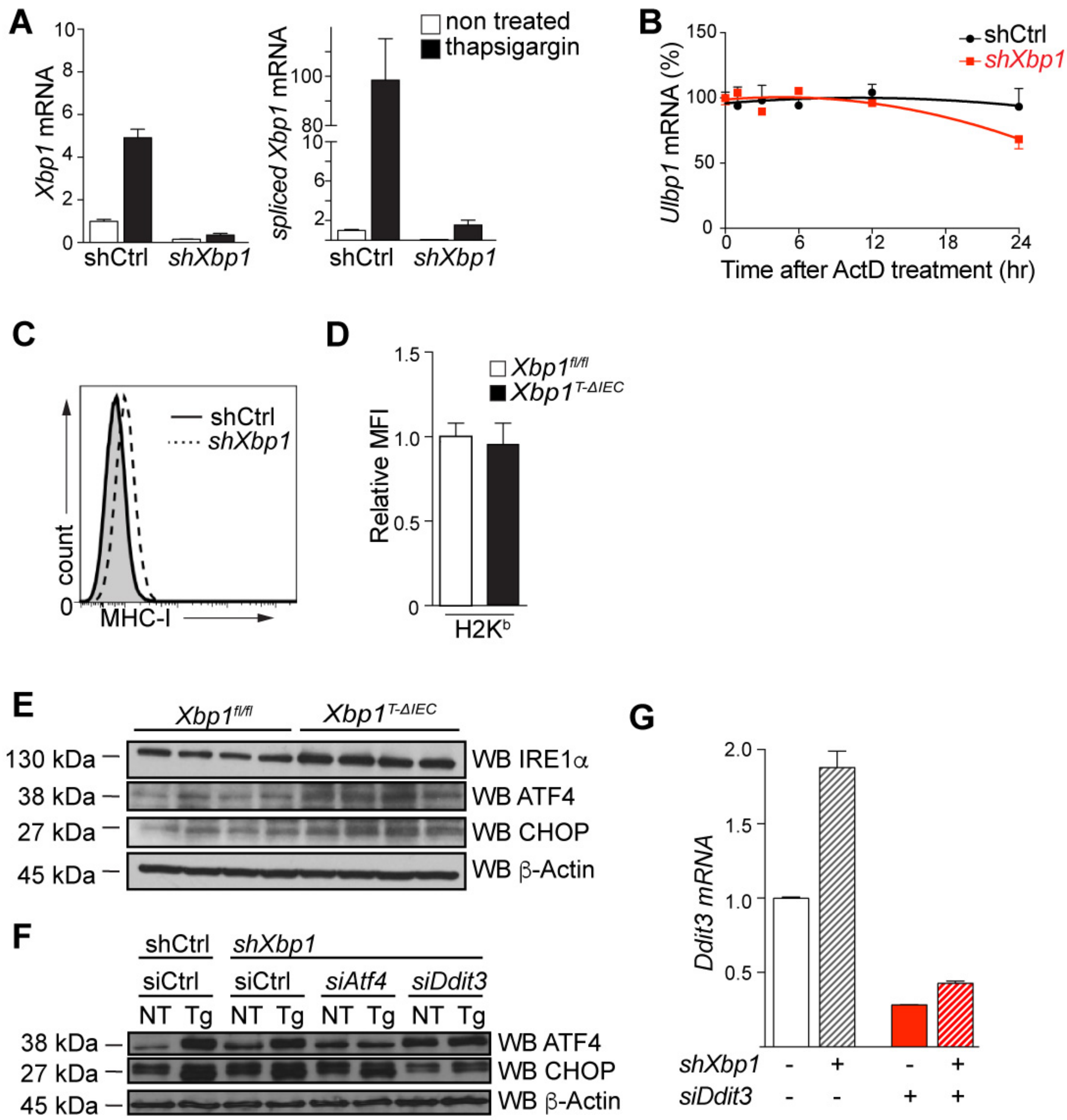


Figure. 5





**Fig. S1**



**Fig. S1**

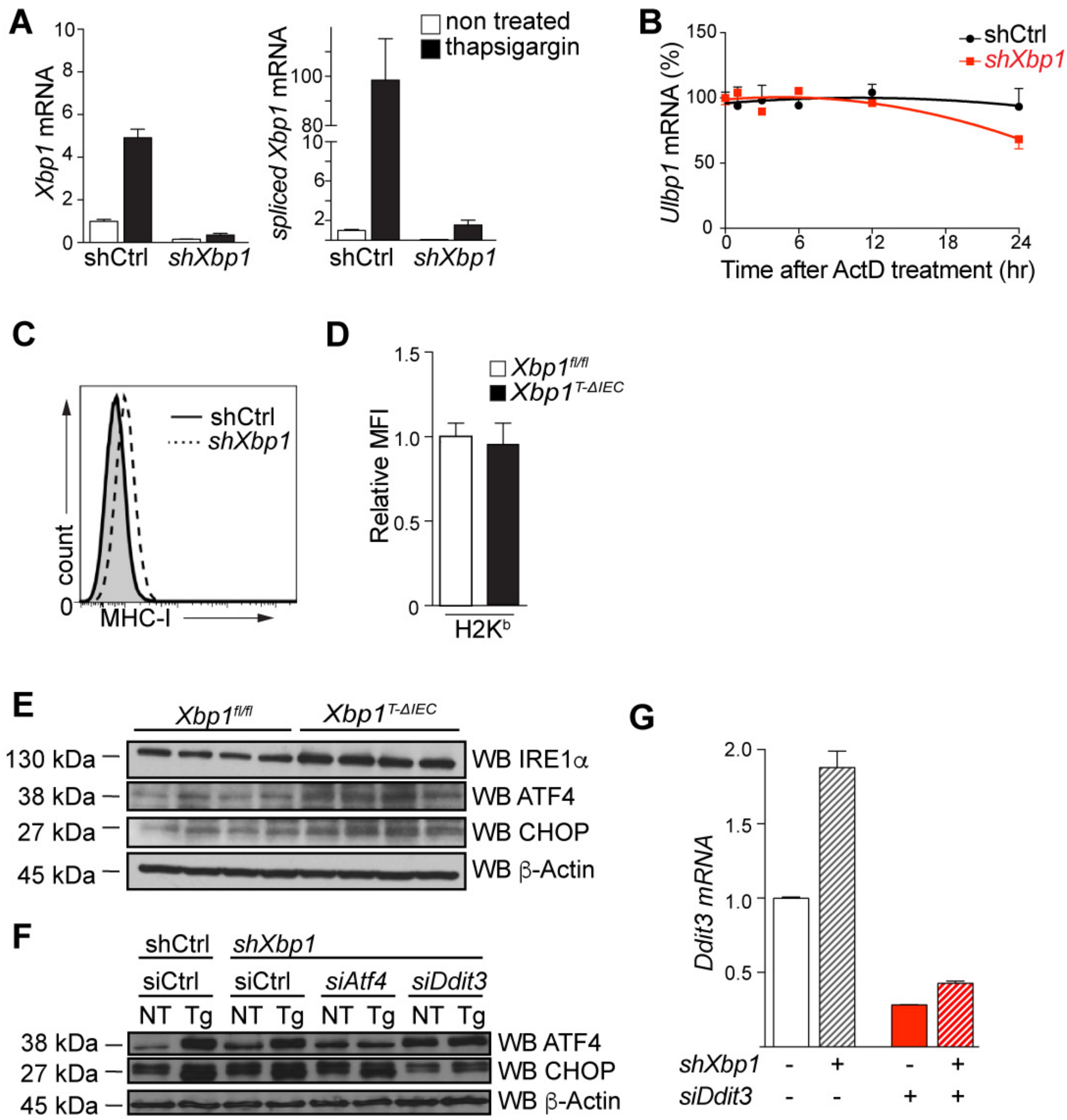
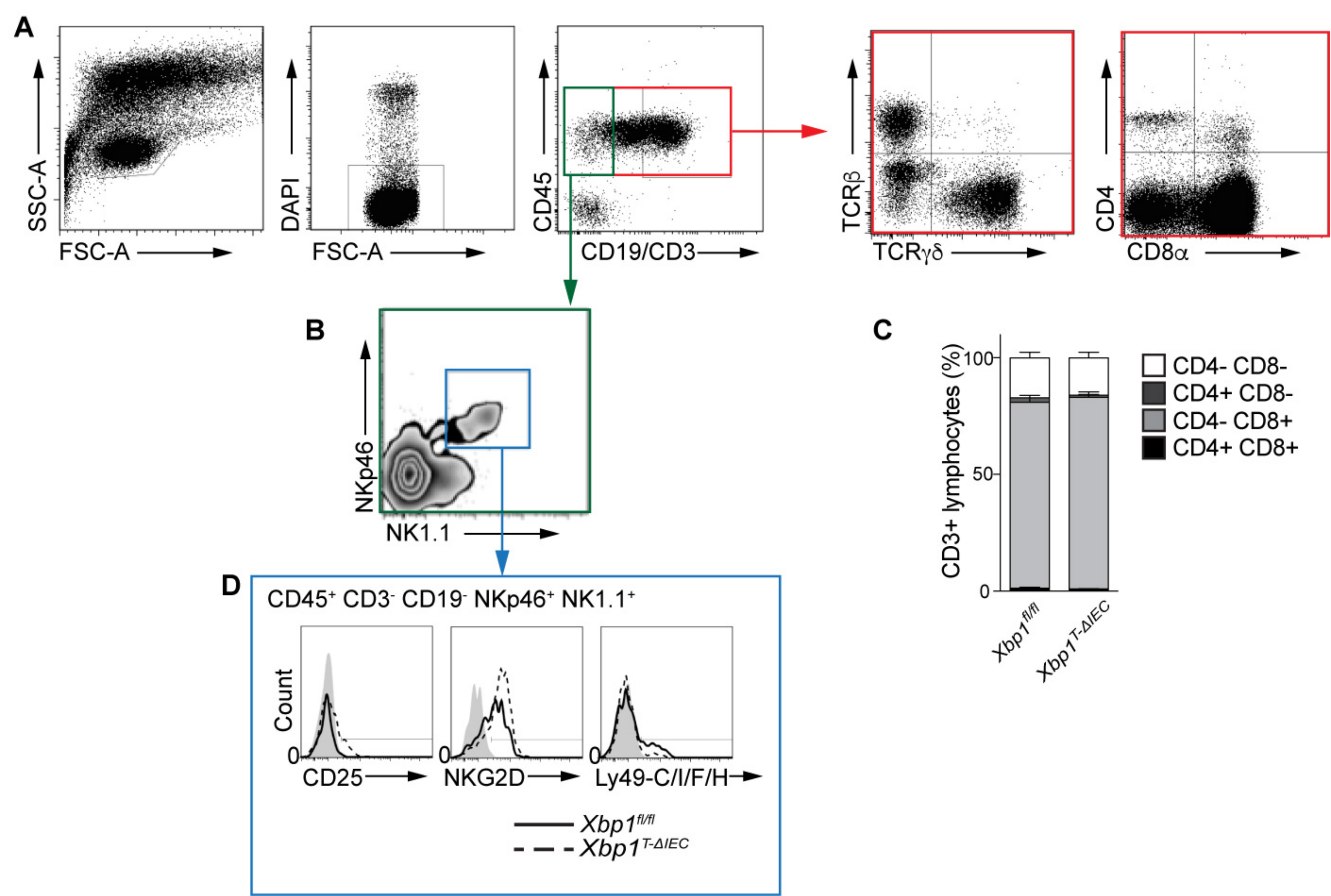
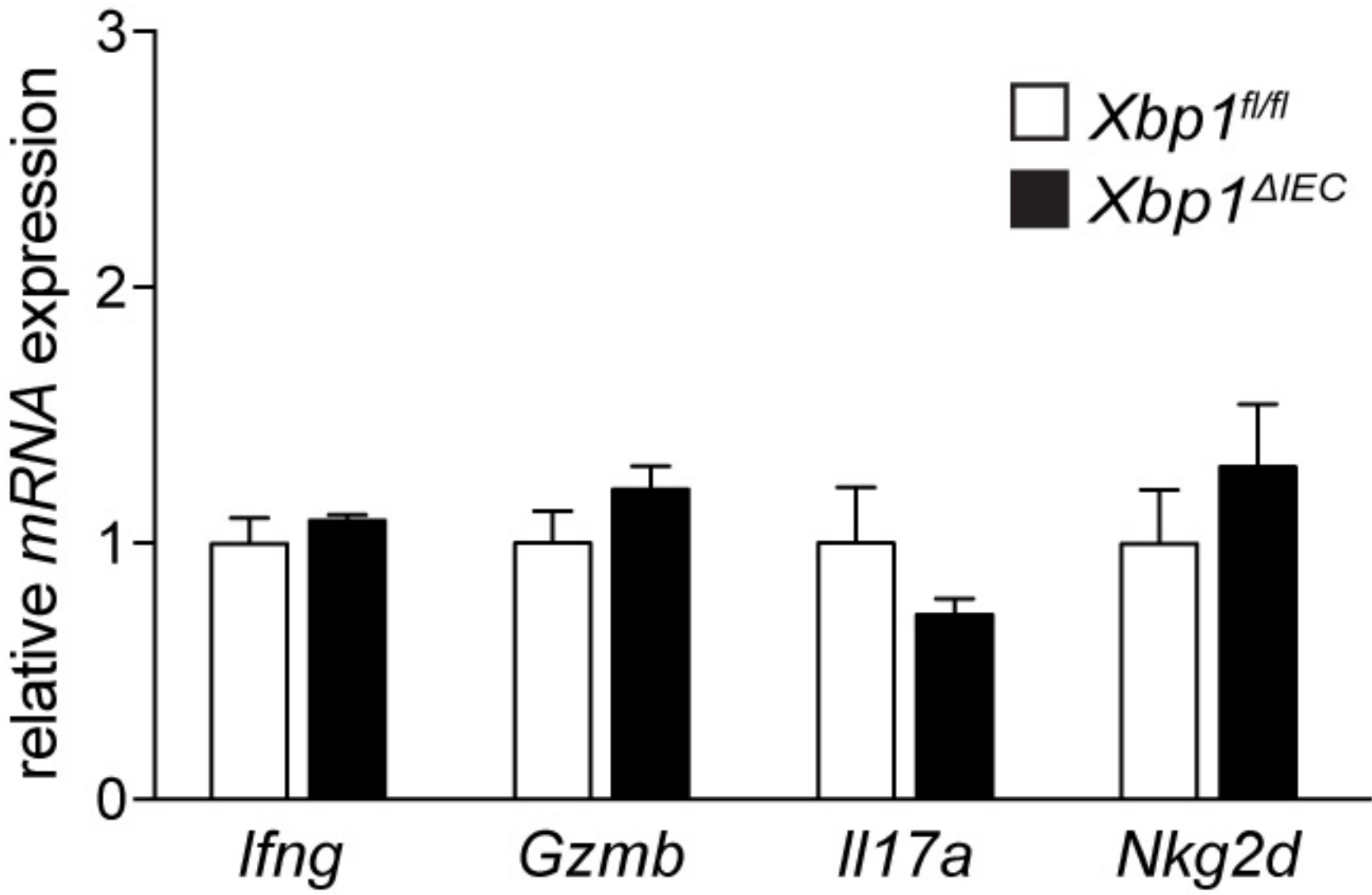




Fig. S2



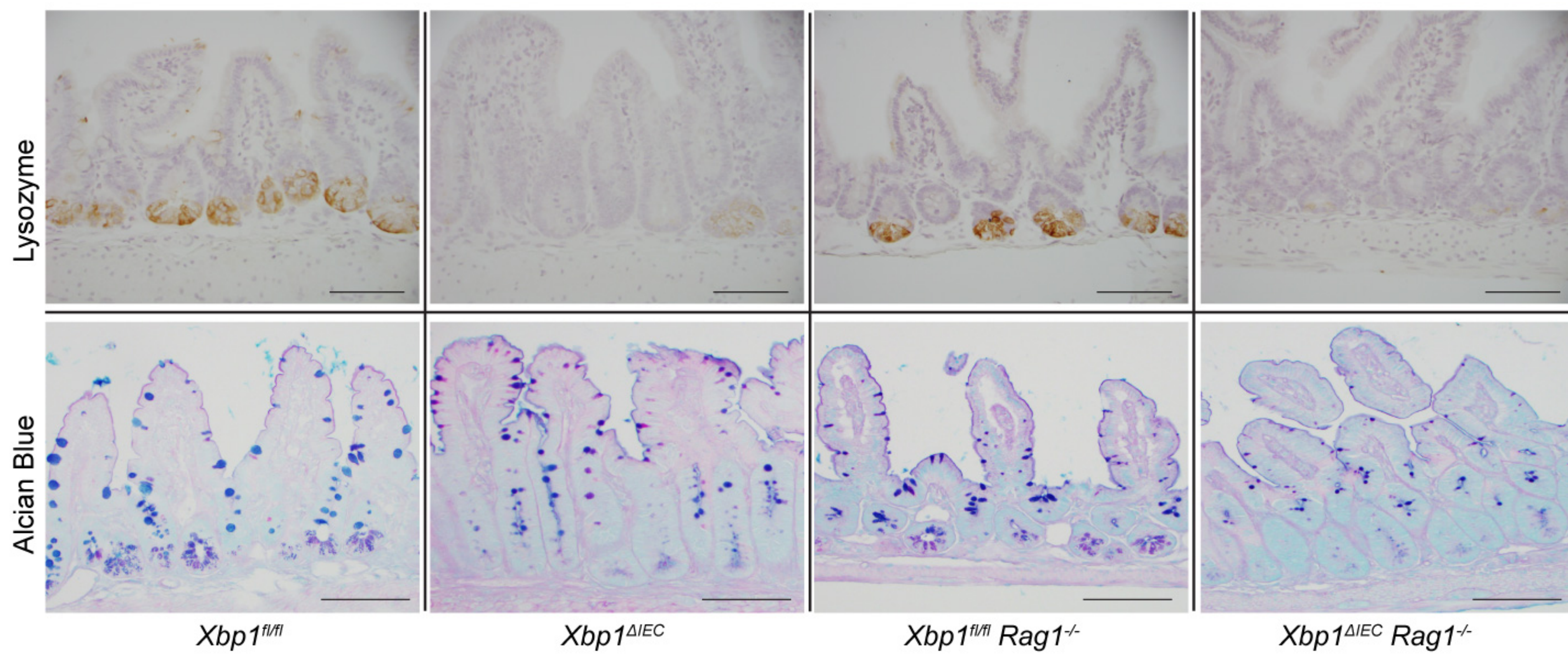
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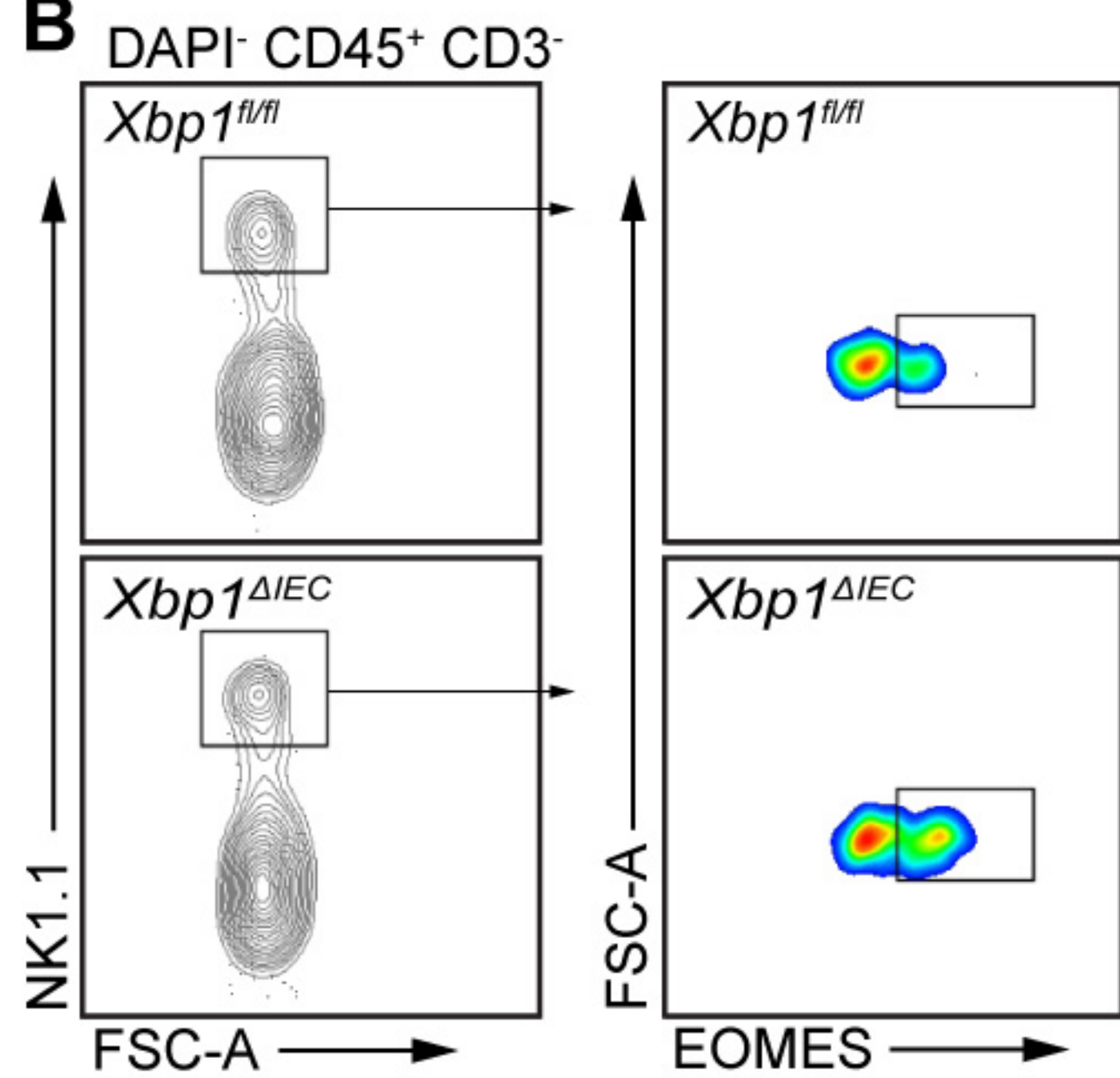


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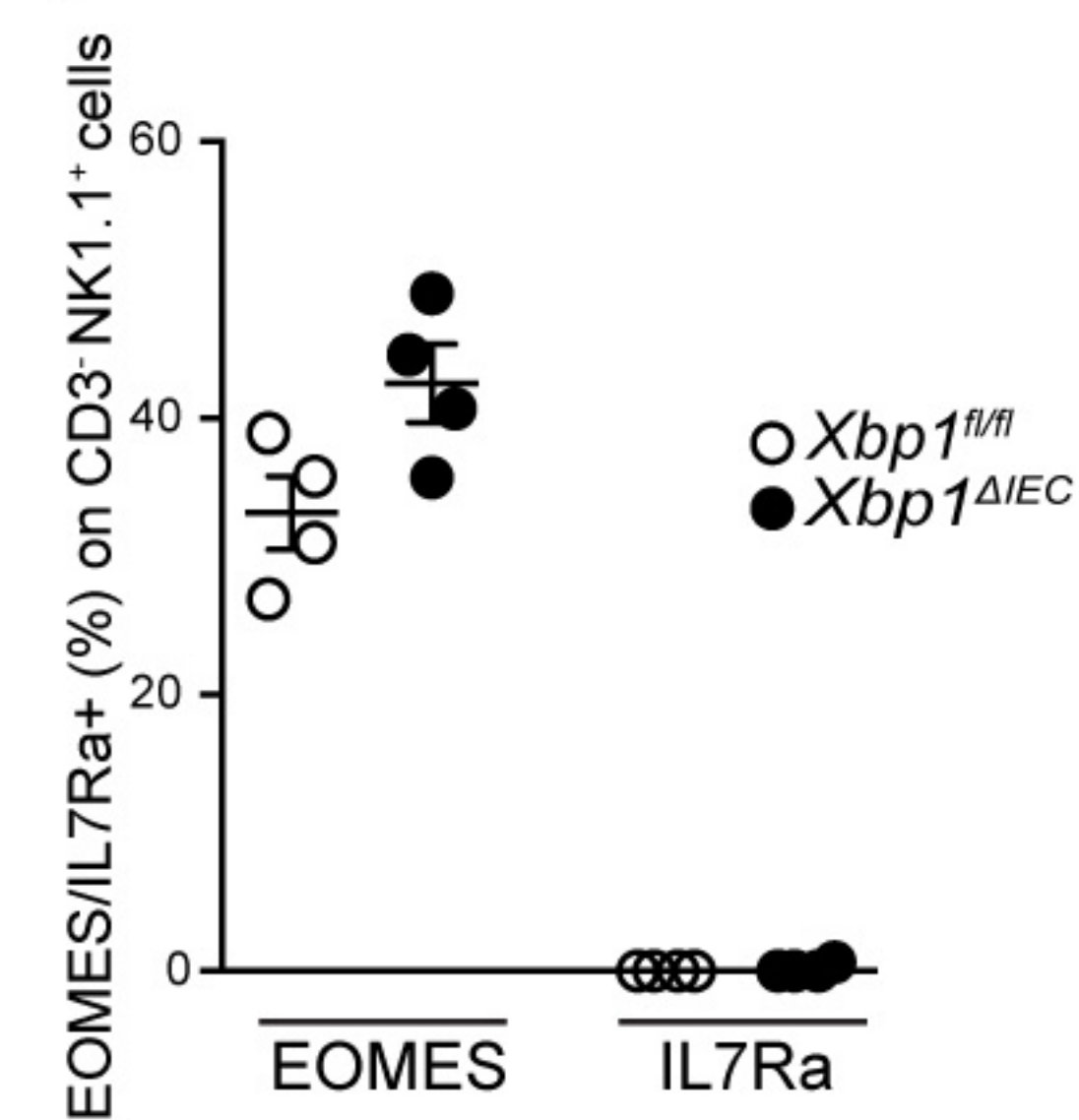
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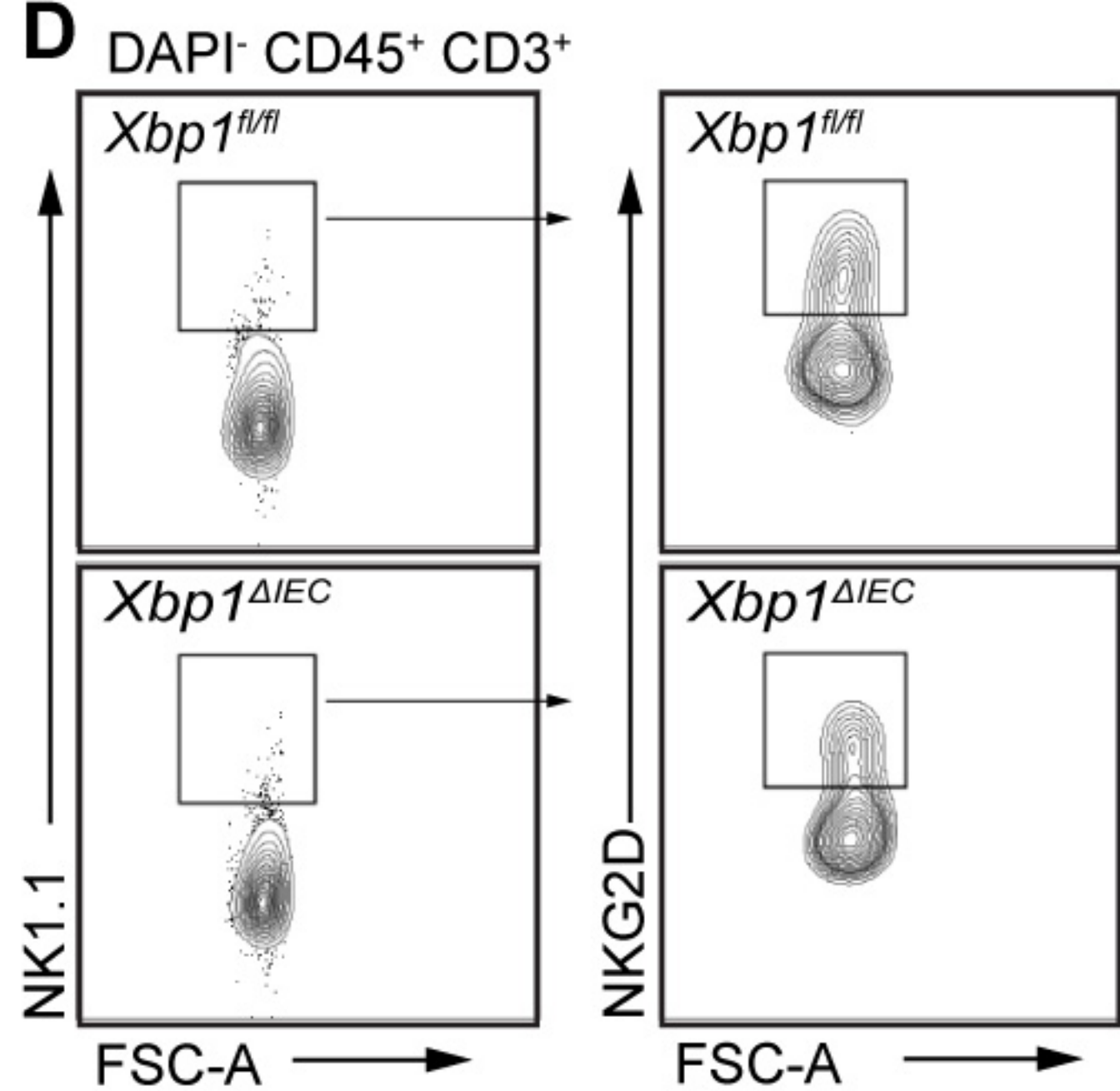
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**C**



**D**



**E**

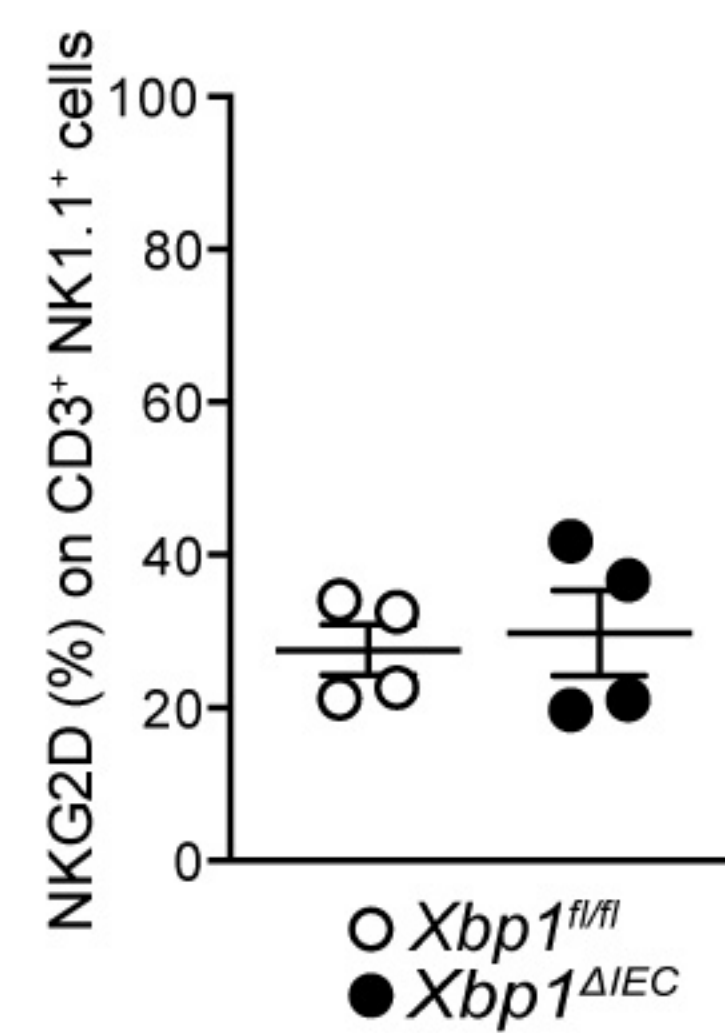




Fig. S5

